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ENVIRONMENTAL SURVIVAL, MILITARY RELEVANCE, AND PERSISTENCE OF *BURKHOLDERIA PSEUDOMALLEI*

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14. ABSTRACT
Burkholderia pseudomallei causes melioidosis, a fatal septicemic infection following either soil or water exposure. The organism is endemic in Asia, where it is able to survive in either desiccated environments or distilled water for several years. The goal of this work was to evaluate available data and to assess the biological hazard posed by *B. pseudomallei* in environmental water, soil, or on inanimate surfaces. We reviewed the historical impact of melioidosis on the military, clinical aspects of the disease, its laboratory diagnosis, and the molecular and phenotypic characteristics of *B. pseudomallei.* We then evaluated the effect of geographic environments and habitats (water, soil, climate), as well as physical (ultraviolet radiation, temperature) and chemical (pH, chlorine) factors on the survival of *B. pseudomallei*. In addition, we analyzed a variety of biological properties that enhance the survival of *B. pseudomallei,* including metabolic adaptation, biofilm formation, and intracellular survival in protozoa, fungi, and specific parts of legume roots. We identified critical data needed for accurate risk prediction and effective threat reduction of the risk posed by B. pseudomallei. The lack of a vaccine, together with its unusual resistance in the environment, makes *B. pseudomallei* a concern for public health and bio-defense.

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SUMMARY

Burkholderia pseudomallei causes a fatal septicemic infection following either soil or water exposure. It is able to survive in either desiccated environments or distilled water for several years. *B. pseudomallei* has been shown to internalize in amoebic cysts and form biofilms, which possibly explains its prolonged persistence in the environment. The goal of this work was to identify and evaluate available data and to assess the biological hazard posed by *B. pseudomallei* in environmental water, soil, or on inanimate surfaces. We reviewed clinical aspects of the disease, its laboratory diagnosis, and the molecular and phenotypic characteristics of *B. pseudomallei.* We then evaluated the effect of geographic environments and habitats (water, soil, climate), as well as physical (ultraviolet radiation, temperature) and chemical (pH, chlorine) factors on the survival of *B. pseudomallei.* In addition, we analyzed a variety of biological properties that enhance the survival of *B. pseudomallei,* including metabolic adaptation, biofilm formation, and intracellular survival in protozoa, fungi, and specific parts of legume roots.

While reviewing the factors affecting *B. pseudomallei*'s environmental survival, we identified critical data gaps that must be bridged for accurate risk prediction and effective threat reduction. There is no vaccine available against human melioidosis. The lack of a vaccine, together with its unusual resistance in the environment, makes *B. pseudomallei* a concern for public health and bio-defense.

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PREFACE

This work described in this report was authorized under the Domestic Preparedness Program. The work was started in March 2005 and completed in December 2006.

Some of the data regarding environmental and general characteristics of *B. pseudomallei* have been summarized in Applied and Environmental Microbiology (Inglis and Sagripanti, vol 72 (11) 6865-6875, 2006).

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ENVIRONMENTAL SURVIVAL, MILITARY RELEVANCE, AND PERSISTENCE OF *BURKHOLDERIA PSEUDOMALLEI*

1. INTRODUCTION

Burkholderia pseudomallei causes a potentially fatal septicaemic infection following either soil or water exposure (Inglis, 1999). The Centers for Disease Control (CDC) listed this organism as a potential bioterrorism agent (www.bt.cdc.gov/agent/agentlist.asp) in 2005. *B. pseudomallei* is notable for its ability to survive in either desiccated environments or distilled water for prolonged periods up to 3 years (Wuthiekanun, 1995). Recently, this pathogen has been shown to internalize in amoebic cysts and can form biofilms (Inglis, 2000); properties that may be critical to the increased persistence of *B. pseudomallei* in the environment. *B. pseudomallei* also appears to adopt a viable, but nonculturable (VBNC), state under conditions of environmental stress (e.g., reduced pH). We have developed a novel selective medium to improve the recovery of *B. pseudomallei* from environmental samples (Inglis, 2003). Ultrastructural changes that are similar to those noted in *Vibrio cholerae* (Morris, 1996) have been observed. Howard and Inglis (2003) describe chlorine-tolerant strains of *B. pseudomallei.* Studies on the effect of chlorine on *Legionella* species and *Burkholderia cepacia* indicate that internalization in amoebic cysts will render *B. pseudomallei* more resistant to chlorine (Kilvington, 1990; Marolda, 1999). Although a candidate vaccine is under trial for glanders (Warawa and Woods, 2002) that could provide some melioidosis protection by cross-reactivity, there is no commercial vaccine available to protect against melioidosis. The combined lack of an effective vaccine and the unusual survival characteristics in the environment make *B. pseudomallei* a significant organism in public health and bio-defense.

2. IMPACT OF THE ENVIRONMENTAL SETTING ON INFECTION (MELIOIDOSIS)

Melioidosis is a potentially fatal bacterial infection caused by *B. pseudomallei*, following inhalation, inoculation, or possibly ingestion of either soil or surface water contaminated by this pathogen (Inglis *et al.,* 1999). Person-to-person transmission has been reported only rarely (McCormick *et al.,* 1975; Kunakorn *et al.,* 1991). There is still some uncertainty as to the most effective route of acquisition. In Australia, a history of a percutaneous inoculation event can be determined in at least 25% of the cases (Currie *et al.*, 2000). Supported by other circumstantial evidence such as the high prevalence of melioidosis in helicopter winchmen during the Vietnam conflict, the pulmonary component of many cases of septicaemic melioidosis has been used as an argument for inhalational exposure. The case for occasional transmission by ingestion is speculative but cannot be excluded completely based on current epidemiological evidence. The rarity of person-to-person transmission reflects the environment's importance in disease pathogenesis and emphasizes the status of humans as dead-end hosts for the pathogen. The minimum infective dose for humans has not been calculated. In the C57BL/BALBc mouse model of melioidosis, the outcome of infection is dose-dependent (Hoppe *et al.*, 1999). When given intravenously, the LD_{50} for melioidosis was between 10^3 and 10^5 bacteria, depending on which strain of mouse was used. However, the median lethal dose was 19 colony forming units in another study that used Porton outbred mice and Syrian hamsters (Russell *et al.*, 2000).

The results of these and other studies suggest that the minimum infective dose will be determined by a range of factors, including bacterial strain virulence , encounter route, host species, and host's physical condition.

The course of melioidosis varies from rapidly progressing septicaemia either with or without pneumonia to localized soft tissue infection, and subclinical infection with delayed conversion to clinically evident infection (Sanford and Moore, 1971; Leelarasamee and Bovornkitti, 1989). Central nervous system infection has a particularly high mortality rate but is uncommon, especially outside Australia (Currie *et al.*, 2000). This may reflect an ascertainment bias due to easy access to tertiary referral hospitals in Australia; but, it could also be explained by either differing genetic susceptibility or differences in the nature of pathogen encounter. There is insufficient data to resolve this issue at present.

There are two variants of *B. pseudomallei* lipopolysaccharide, which are thought to be responsible for the clinical and pathophysiological features of septicaemic melioidosis. Several toxins have been proposed; but their role in clinical infection is controversial. Fatal disease occurs mainly in people with major co-morbidities: diabetes, chronic renal failure, and alcoholic liver disease. It is notable that these have a metabolic acidosis with raised levels of ketone bodies (β-hydroxybutyrate), leading investigators to speculate that septicaemic disease progression might be the result of substrate use by *B. pseudomallei* (Inglis, 2002).

3. DETECTION AND LABORATORY DIAGNOSIS

The nonspecific nature of the clinical features of melioidosis forces the clinician to rely on the laboratory to diagnose the disease. Isolating *B. pseudomallei* from clinical samples is the standard against which all other methods are judged. The recovery of a gram negative bacillus with characteristic phenotypic features may alert laboratory staff to the diagnosis. But, the shortcomings of commonly used bacterial identification systems can produce misleading results and contribute to a missed or delayed etiologic diagnosis (Inglis *et al.*, 1998). The current recommendation is to use genotypic confirmation of bacterial identity [e.g., a *B. pseudomallei*-specific nucleic acid amplification protocol (Inglis, 2003)]. A rise in antibody titre may either suggest or support the etiologic diagnosis, but is unlikely to help in the early stages of a septicaemic process. Serological tests for melioidosis are more useful for epidemiological studies and possibly in following the course of disease during attempts to fully eradicate infection from the patient (Vasu *et al.*, 2003).

4. ENCOUNTERS WITH ENVIRONMENTAL *B. PSEUDOMALLEI*

Epidemiological data indicates that the principal means of acquisition is occupational or recreational exposure to *B. pseudomallei* (Leelarasamee and Bovornkitti, 1989). The individuals most commonly affected are rice farmers, servicemen, miners, adventure travelers, and indigenous peoples in the main endemic zone of northern Australia and Southeast Asia (Figure 1). Recently, the disease has been recognized in Central and South America, where a lack of advanced diagnostic services may cause the infection to be under-diagnosed or missed entirely (Miralles *et al.*, 2004).

There is a long and growing list of countries in which melioidosis is thought to be endemic (Leelarasamee and Bovornkitti, 1989). The main endemic zone is in Southeast Asia and northern Australia. Cases have also been confirmed by culture of *B. pseudomallei* from the Indian Subcontinent, the Middle East, and South and Central America. Most patients were exposed between latitude 20°N and 20°S. With the exception of one controversial case from Oklahoma, there is no evidence in North America for local acquisition of melioidosis outside the laboratory. The epidemiology of melioidosis in Africa remains uncertain, and may reflect an almost universal lack of relevant experience and advanced diagnostic laboratory infrastructure. There is documented human melioidosis in the Hong Kong special autonomous region (SAR) and parts of southern China, including Hainan Island (Figure 2). The status of the disease in North Korea is unknown. There were well-documented human cases of melioidosis in Iran in the 1970s. Although none have been confirmed in Iraq, an adult female civilian visiting southern Iraq late in 2003 experienced a severe seroconversion illness and is thought to have had melioidosis (Inglis, unpublished laboratory data). Melioidosis was among the differential diagnoses considered for the acute respiratory syndrome noted in armed forces serving in Iraq in mid 2003 (Inglis, ProMed 10-AUG-03; 20030810.1978). Recently, in the Americas, melioidosis has been recognised as an emerging infectious disease in Brazil (Figure 3). The isolate recovered during the initial case cluster investigation was similar to the West Australian outbreak strain (now, *B. pseudomallei* NCTC 13177). Now that Brazilian authorities have developed a case definition, additional cases of clinically suspected melioidosis are being discovered more widely. Their confirmation will depend on the development of diagnostic laboratory capability. It is likely that the disease will turn out to be widespread in Brazil and other parts of tropical South America. Melioidosis has been reported from Puerto Rico (Dorman *et al.*, 1998). There have been unpublished, anecdotal reports of melioidosis in Venezuela and El Salvador (Champaigne, correspondence, 2001). The last source location only came to light through investigation of infections in refugees arriving in the United States.

5. MELIOIDOSIS AND THE MILITARY

Melioidosis was first reported by army medical staff in Burma in 1912 (Whitmore and Krishnaswami, 1912). Shortly afterwards, there were cases of disease in laboratory animals from Malaya where an epizootic in a research animal house led to speculation that melioidosis was zoonotic (Stanton and Fletcher, 1925). This focus on the role of animals in the disease and the need to distinguish it from glanders led veterinary scientists to document melioidosis in a series of domestic animal species. There were few additional reports of human infection until military physicians recognised the infection during the conflict in French Indochina. Careful investigation highlighted the possible role of *B. pseudomallei*-contaminated soil as a source of the infective agent (Chambon, 1955). Melioidosis was recognized with increasing frequency as a Southeast Asian disease during the Vietnam conflict, when servicemen contracted the infection during their tours of duty. A particularly high incidence was noted among helicopter winchmen, leading to the proposal that inhalation of an infective aerosol was an important means of transmission (Howe *et al.*, 1971). There are anecdotal reports of approximately 1000 cases dealt with by one military hospital, and up to several hundred servicemen under treatment for melioidosis at any one time (Smith, correspondence, 2000). Years after the end of the Vietnam conflict, it became clear that melioidosis had a capacity to remain dormant for prolonged periods, the longest recorded being around 29 years after presumed exposure in the theater of operations (Chodimella *et al.*, 1997). A late onset case of cutaneous melioidosis following presumed

exposure in the Pacific Theater during the second world war has been recently reported – a likely disease-free interval of 62 years (Ngauy *et al.*, 2005). Late onset septicaemic infections are thought to have occurred due to physiological stress (e.g., the induction of a general anaesthetic in patients with sequestered disease). Interestingly, seroepidemiology studies in British special forces deployed in the endemic region during the Malayan Emergency did not show evidence of residual infection (Preston, 1976). Moreover, late onset melioidosis has not been a problem in Australian veterans previously deployed in Vietnam. It is not clear why U.S. forces contracted this disease when allied units deployed on similar operations in the endemic region, close to locations where the native population currently experiences melioidosis, did not contract the disease.

6. ENVIRONMENT AND EPIDEMIOLOGY

The likely aerosol or dust-borne mode of transmission does not answer key questions such as how long the bacteria can survive in an infectious form, and what is the infective dose? The lack of a reliable method for enumerating bacteria in a viable but nonculturable state in either soil or water specimens makes these questions difficult to tackle. Moreover, it is as yet unclear whether survival in the environment makes *B. pseudomallei* either more or less virulent.

Recent epidemiological studies have shown alternative vehicles for transmission and have extended the area considered endemic for melioidosis. The sharp increase in acute septicaemic cases in the Darwin in 1990-91 mainly affected Australian aboriginal people. After a higher incidence of co-morbidities (e.g., diabetes and chronic renal failure, and a high level of exposure to moist surface soil were considered. There was no independent risk attributable to ethnic status (Merianos *et al.*, 1993). A series of investigations into a case cluster in northwestern Australia in late 1997 implicated the drinking water supply to the affected town (Inglis *et al.*, 1999) and documented a series of deficiencies in the drinking water system, including contamination of an aerator of a water installation, low available chlorine levels, and an unusually high drinking water demand (Inglis *et al.*, 2000b). Further instances of water supply-related melioidosis have since been documented (Currie *et al.*, 2001). Unpublished data from Australia indicates that sporadic cases and seasonal peaks of septicaemic disease are usually associated with soil exposure, while case clusters appear to be linked with water contamination. Despite the detailed epidemiology conducted in Australia and parts of Southeast Asia, it is unclear whether the final route of exposure to contaminated water is by inhalation, percutaneous inoculation, or ingestion.

The recent case cluster in Northern Brazil was associated with the onset of unusually heavy rainfall during which the four children involved swam off a recently replenished irrigation dam (unpublished data). Australian data indicates that the peak risk of septicaemic disease is in the 2 weeks following the onset of summer rainfall in the tropical north (Currie and Jacups, 2003). In the Hong Kong SAR, the peak risk for cases of melioidosis in marine mammals is around the time of the typhoon. Recent unpublished data suggests that *B. pseudomallei* may be washed out of rain clouds; however, this observation has yet to be supported by systematic sampling.

Careful prospective environmental sampling across northern Australia targeted around the location of septicaemic cases has shown that the environment is neither widely nor heavily contaminated throughout the endemic region, despite assertions repeatedly made by public health authorities. Culture-positive environmental samples of surface soil and water were surprisingly rare (unpublished data). However, polymerase chain reaction (PCR)-positive samples have been obtained from sites where cultures were persistently negative (Brook *et al.*, 1997). A prospective analysis of one industrial site in northern Australia has obtained PCR-confirmed *B. pseudomallei* only from one small cluster of locations and then only during the peak of the wet season (Inglis, unpublished data). Seroconversion and culture positive infection have been documented in people working at this location. The type and frequency of exposure to *B. pseudomallei* has yet to be determined conclusively. But, an outline estimate of seroconversion risk in the European population in this region is around 3-5% per annum.

The indigenous population of the Australian endemic region has a higher prevalence of antibodies to *B. pseudomallei* (Ashdown and Guard, 1984), probably reflecting a higher level of exposure to contaminated soil and water. In Southeast Asia, these antibodies may be a reaction to exposure to the nonpathogenic *B. thailandensis*. As *B. thailandensis* has not been isolated from Australia despite prolonged attempts to do so, it is unlikely that this high seroprevalence in some Australian populations represents a cross-reaction to Southeast Asian nonpathogenic *Burkholderia* species. Nevertheless, it is possible that low level exposure to environmental *B. pseudomallei* may render the indigenous population relatively resistant to melioidosis. What is clear from occupational health studies is that fit, healthy Europeans can develop septicaemia, soft tissue infection, or maybe seroconvert. Although the majority of serious infections are in people with underlying co-morbidities, there have been several recent cases in previously fit people in Australia. There have been either fatalities or near-fatalities from septicaemic melioidosis in fit males, emphasizing the importance of either host factors (e.g., genetic predisposition) or pathogen factors (e.g., either unusually high virulence or large inoculum). Examination of autopsy tissue samples from one such fatality showed a predilection of *B. pseudomallei* for human cells with a specific lectin-binding site (Inglis, unpublished data). It has not yet been possible to confirm this preliminary observation for lack of further autopsy material. However, the case concerned involved exposure to mud pools in northern Australia, judging from the video diary the victim kept.

The infectious dose of *B. pseudomallei* has yet to be determined for humans. A differential course of infection has been demonstrated, depending on the mouse strain employed as model, with BALBc mice being much more susceptible to septicaemia than C57 mice (Ulett *et al.*, 2000). There are additional differences attributed to whether the infectious challenge is intraperitoneal (high dose inoculum required) or intranasal (lower inoculum). Bacterial inoculum density and growth phases are both important to cellular invasion in laboratory model systems (e.g., *Caenorhabditis elegans* and *Acanthamoeba astronyxis*) (O'Quinn *et al.*, 2001; Inglis *et al.*, 2000). For this reason, melioidosis results from a mouse model may not translate directly to a human setting. The mouse model has been used for experiments on stationary phase *B. pseudomallei.* No data has been published on attempts to repeat the same experiments with bacteria synchronized to either the lag or log phases of growth, de-synchronized bacteria (as might occur in a real-life environment), or bacteria passed via a free-living phagocyte such as Acanthamoeba.

The limited data on laboratory-acquired infections indicate that a high infectious dose is probably required to establish infection in a healthy worker (Ashdown, 1992). However, routine handling of laboratory plates bearing *B. pseudomallei* may result in low level seroconversion (Currie *et al.*, 2004). In Australia, *B. pseudomallei* is categorised as a biosafety level 2 pathogen, despite recommendations that it be handled at BSL 3 (Ashdown, 1992). However, aerosol-generating procedures are carried out with additional precautions in a class 2 biosafety cabinet. In jurisdictions with less experience of melioidosis, *B. pseudomallei* is handled at BSL 3. There are no recent data on seroprevalence among diagnostic microbiology laboratory workers in Australia.

7. PREVENTION AND TREATMENT

The mainstay of melioidosis prevention is avoiding exposure. Personal protective equipment can be effective where the location and timing of exposure is predictable (e.g., servicemen on exercise in a known disease hot area). Clearly, this is not the case when considering most sporadic cases of acute septicaemic disease. Cleaning cuts and superficial abrasions to reduce the effect of either soil or surface water contamination may also help prevent melioidosis (Inglis *et al.*, 1999), though there is no data on how effective this might be.

There is no commercial vaccine available for human use. The nearest candidate is a flagellin-glycopeptide conjugate subunit vaccine under trial for glanders in large animals (Warawa and Woods, 2002). There is sufficient cross-reactivity with *B. pseudomallei* to expect a protective effect against melioidosis when safety trials have been completed. But it will be some time before safety and efficacy trials have been completed in at-risk Europeans who have not yet been exposed to *B. pseudomallei*. Moreover, the intracellular nature of melioidosis makes stimulation of T cell immunity difficult. Therefore, a vaccine to provide complete immunological protection against melioidosis is unlikely. There are other candidate vaccines under consideration, including a possible DNA vaccine against *B. pseudomallei*. These are at a much earlier stage of development; and it will take much longer to bring them to operational readiness. No work has been published yet comparing the differential susceptibility of melioidosis-naïve and melioidosis-exposed populations. Although the epidemiological data points to soil/water exposure and underlying comorbidities rather than ethnicity as the main determinants of infection, it remains to be seen whether more subtle immunogenetic characteristics modulate the humoral response to infection. There is some evidence in humans that there may be a genetic basis to cell-mediated immunity to melioidosis, as one might expect from an intracellular infection. A study in Thailand showed a higher frequency of specific histocompatibility locus antigens (HLA) DR alleles in melioidosis, and the more severe forms of the disease (Dharakul *et al.*, 1998). How this translates to a Caucasian healthy adult group, at-risk only through environmental exposure, is an important, unresolved issue.

Melioidosis treatment can be considered in three phases: septicaemic, localized soft tissue, and subclinical. Septicaemic disease, either with or without pneumonia, needs to be treated with intravenous antibiotics. Ceftazidime is widely used and was the first antibiotic shown to significantly reduce the mortality of septicaemic melioidosis (White *et al.*, 1989). More recently, carbapenems have been found to be at least as effective and may have superior intracellular effect (Simpson *et al.*, 1999; Cheng *et al.*, 2004;

Inglis *et al.* 2004). Prospective clinical trials have not been continued long enough to determine whether the carbapenems are superior to ceftazidime in terms of clinical outcomes. Investigators think that a combination of ceftazidime and co-trimoxazole is better than ceftazidime alone. There is a well-recognised risk of septicaemic relapse between weeks and months of commencement of antibiotic treatment. In some studies, this relapse has been around 23% (Chaowagul *et al.*, 1993). To prevent relapse, continuation of oral-suppressive antibiotic therapy for several months after initiation of treatment is considered essential. A consensus is forming around the need for several oral agents to be administered in combination. When several of these agents are given together, so-called maintenance therapy can be truncated to around 3 months. But, some authorities believe that in patients with significant co-morbidity risks (e.g., diabetes), lifelong suppressive therapy may be needed. Difficult questions remain regarding post-exposure prophylaxis where there is no clear consensus. Co-trimoxazole alone or plus doxycycline have been used for this purpose. But, the combination appears to be superior only to prevent relapse. The optimal duration of therapy and monitoring are unclear (Chaowagul *et al.*, correspondence).

8. CHARACTERISTICS OF *B. PSEUDOMALLEI*

B. pseudomallei is an oxidase positive, gram negative bacillus that was transferred to the new genus *Burkholderia* formed by members of the former pseudomonas RNA homology group II (Yabuuchi, 1992). The recently published genome of *B. pseudomallei* strain K96243 contains two chromosomes of 4.07 megabase pairs and 3.17 megabase pairs, respectively (Holden *et al.*, 2004). The large chromosome encodes many core functions associated with central metabolism and bacterial growth. The small chromosome carries more genes associated with adaptation and survival in different niches. Genomic comparisons with related bacteria suggest that the two replicons have distinct evolutionary origins. Another feature of the *B. pseudomallei* genome was the presence of 16 gene islands that made up 6.1% of the genome, which were present in clinical and soil isolates but entirely absent from the clonally related organism *B. mallei*. Mature colonies may take on a wrinkled appearance after several days' incubation on solid media (Pitt, 1995), though this wrinkling affect is lacking in a proportion of strains and is more pronounced on some solid agar formulations containing glycerol (Howard and Inglis, 2003a). Some strains of *B. pseudomallei* produce smooth colony growth on first culture, and occasional strains are overtly mucoid with an appearance similar to *Pseudomonas aeruginosa* capsular polysaccharide over-producers (Rogul and Carr, 1972) (Figure 4).

B. pseudomallei uses a wide range of substrates, including a variety of sugars in assimilation reactions (Pitt, 1995). An important exception among these is L-arabinose, which the closely related but nonpathogenic *Burkholderia thailandensis* can metabolize (Smith *et al.*, 1997). This critical difference in L-arabinose use is most commonly used to distinguish between these two species, although a wider range of substrate metabolization reactions distinguish them apart. There are also relatively few differences in substrate-utilization patterns between *B. pseudomallei* and *Burkholderia cepacia*; the opportunist pathogen of cystic fibrosis patients. Laboratory identification systems that rely on substrate-utilization profiles often erroneously label *B. pseudomallei* as *B. cepacia* (Inglis *et al.*, 1998; Inglis *et al.*, 2005).

Like other members of the genus *Burkholderia*, *B. pseudomallei* accumulates polyhydroxybutyrate (PHB) in large, central granules to give a negative staining effect on

gram stain (Pitt, 1995). The appearance is often one of bipolar staining, though this is not specific to the *Burkholderias* and may be absent in young colonies. The bacillus has one or more terminal flagella and shows motility, particularly in the early stages of its growth cycle.

9. DETECTION AND DIAGNOSTICS

Given the above difficulties in identifying *B. pseudomallei*, diagnostic laboratories that often encounter the species have turned to alternative methods for definitive identification. Several hospitals and research centers use an in-house agglutinating antiserum for rapid confirmation (Smith *et al.,* 1993). A latex-agglutinating monoclonal antibody test is now commercially available. This antibody correctly reacted with 85% *B. pseudomallei* isolates and 28% other closely related *Burkholderia* species (Inglis *et al*., 2005). Other groups have used latex agglutination for diagnostic purposes, but have not progressed to commercialization of their reagents. The loss of sensitivity is due in part to the practical difficulty of operating a latex agglutination test on a glass microscope slide behind the glass of a class 2 biosafety cabinet. Nucleic acid amplification of *B. pseudomallei*-specific sequences is another approach that has been successfully used. The first published and most widely used protocol relies on a portion of the 16s-23s spacer region (Kunakorn and Markham, 1995). A more recently published alternative target is a portion of the groEL transcriptional regulator, which was used to develop sequencing primers (Woo *et al.,* 2001; Woo *et al.,* 2003). The sequence of the amplified groEL product was used to distinguish between *B. pseudomallei* and the nonpathogenic *B. thailandensis*. We have added protocols based on variable regions from the polyhydroxybutyrate synthesis operon and from a part of an alpha ketoglutarate-dependent dioxygenase gene homologue (Gibbons *et al.,* 2000). Conventional PCR protocols for these targets are now being converted for real-time PCR applications, and their performance is being compared across a collection of *Burkholderia* species strains.

10. ENVIRONMENTAL SURVIVAL CHARACTERISTICS

The unusual ability of *B. pseudomallei* to survive for months to years in the environment can be inferred from its persistence in the melioidosis endemic zone throughout the dry season. It is notable that the ability to survive an adverse soil or water environment appears to parallel the ability of *B. pseudomallei* to sequester in human macrophages and lympho-reticular organs in a dormant or quiescent state. Judging from environmental samples, *B. pseudomallei* does not appear to persist in a culturable form for more than a matter of hours or days. The exception is persistence in culturable form for up to at least 3 years in distilled water (Wuthiekanun *et al.*, 1995). In contrast, nonculturable *B. pseudomallei* probably persists for at least 1 year if it is the form in which the species survives the dry season. Some of the assertions made about the environmental survival of *B. pseudomallei* are based on extrapolations from other bacterial species. These may underestimate the capacity of this species to survive in and adapt to hostile habitats. While some data is available on the survival of B. pseudomallei under a few specific conditions of nutrient, acid, and thermal stress (Table 1), more detailed survival kinetics are required to develop a predictive survival model of the bacterium in the environment.

From our own data, the inactivation kinetics of *B.pseudomallei* in trypticase soy broth (TSB) appear complex. The bacterial response in liquid medium to the different

physical and chemical stresses that we have tested follows a broadly similar pattern. The culturable number of bacteria generally fall very quickly with increasing stress levels, then fluctuate before reaching a steady state lower than the starting count. Only preliminary data is available on survival in different growth media and varying physical conditions (Table 1).

10.1 Bacterial Survival In Water.

Given the ability of *B. pseudomallei* to survive long periods in moist soil, the species' ability to survive in water has been studied at length, though the mechanism of prolonged survival is still not understood. Cultures of *B. pseudomallei* inoculated into triple distilled water have remained culture positive for over 3 years (Wuthiekanun *et al.*,1995). The starting count of 5 $\log_{10}$/mL rose to 8 $\log_{10}$/mL after 1 month and then fell steadily to 4 $\log_{10}$/mL after 2 years (Table 2). We understand that the experiment was continued for several years beyond the publication date and still the cultures remained positive (Wuthiekanun, correspondence). The count of the *B. pseudomallei* strain implicated in the Western Australian outbreak of 1997 dropped from 6 $\log_{10}$ to around 4 $\log_{10}$ colony forming units (CFU)/mL in the 24 hr after its inoculation into filter-sterilized potable water (circa 1 ppm chlorine) from the affected community. However, the count returned to the original count 4 weeks after inoculation. The capacity to survive in distilled water for prolonged periods implies a water bacteria lifestyle in which long-term carbon storage in bacterial cells powers slow-rate metabolism. The PHB granules present in mature, stationary phase bacilli may fulfil this role. No attempt has been made to disable PHB polymerase in *B. pseudomallei* by either silencing or knocking out the genes involved. This function has been studied in *Legionella pneumophila* in relation to aquatic survival (James *et al.,* 1999). Polyhydroxybutyrate utilization corresponded to the decline in culturable bacterial cells (Figures 5 and 6). In view of the fact that all the Burkholderia including nonpathogenic species produce PHB, it is unlikely that PHB synthase is a virulence factor. However, it is important to recognise that mechanisms of cellular virulence in mammalian cells may require sophisticated metabolic survival strategies that have already been rehearsed in a more typical environment or habitat. The expression of *B. pseudomallei* PHB synthase, depolymerase, and beta-hydroxybutyrate dehydrogenase have yet to be investigated in this context.

10.2 Soil Type And Survival.

Periodic soil sampling for *B. pseudomallei* has shown that the persistence of culturable bacteria depends on the soil type, hydration, and starting inoculum (Thomas and Forbes-Faulkner, 1981). Well drained, light sandy soils are less able to support prolonged persistence of *B. pseudomallei*. Waterlogged, heavy clay soils are much better at supporting bacterial persistence. The data was obtained qualitatively before our current quantitative methods had been developed (Inglis *et al.*, 2004). Soil survival curves have not been given priority in medical research funding, so quantitative survival of *B. pseudomallei* in soils is not available. Available qualitative data indicate that a faster decline in bacterial count has been inferred in dryer soil environments (Table 2). Deeper samples (>30 cm) taken in field studies were more likely to contain culturable *B. pseudomallei*, possibly reflecting the higher residual water content and lower depths (Thomas and Forbes-Faulkner, 1981). Thai workers have dug deeper in their softer soils to a depth of 90 cm and have obtained counts of up to 10^5 CFU *B. pseudomallei* per milliliter of soil supernatant, with a median count of 250 CFU/mL in soil from the main endemic region of northeast Thailand (Smith *et al.*, 1995). This work was performed prior to the development of accurate quantitative bacteriology methods and lacks the detail necessary to calculate survival kinetics. No published work has dealt with

B. pseudomallei survival in reconstituted soils in a controlled laboratory setting. A rising water table at the onset of the tropical summer rainy season was cited as an explanation for reappearance of *B. pseudomallei* in the more superficial layers of soil prior to human exposure. In a limited study that presents very little numerical data, soil with a water content of <10% led to the death of *B. pseudomallei* within 70 days, while soil with a water content of >40% maintained bacterial life for 726 days (Tong *et al.*, 1996). This preliminary study does little more than outline the capacity of this species to survive different environmental conditions and does not permit formal calculation of survival fraction, time-dependent kinetics, or water-content dependency. It is possible that the attraction of *B. pseudomallei* to oxygen (aerotactic) leads to preferential colonization of the water-air interface bridging soil particles in well-aerated moist soils. The effects of desiccation on bacterial survival and recovery have not been studied at a bacterial cellular level. Water content is a well-recognised contributor to survival of foodborne bacterial pathogens. Given the capacity of *B. pseudomallei* to survive for months to years in distilled water, study of the survival kinetics of this species in soils of varying water content will assist in predicting melioidosis risk. From what little is known, increased *B. pseudomallei* soil half-life would be expected with increased water content. Other physical characteristics of soil such as particle size, organic content, and hydrophilic/ hygroscopic properties are also expected to have some effect on *B. pseudomallei* survival.

10.3 Bacterial Survival in Dry Environments.

Though not as prodigious as its ability to survive in water, *B. pseudomallei* is more resistant to desiccation than many other gram negative bacilli (Tong *et al.*, 1996). It is not clear why this might be. The explanation may lie in the unusually high peptidoglycan content for a gram negative cell wall (circa 35%), or it may be a reflection of a strong stress-survival response. Whatever the reason, *B. pseudomallei* can be cultured from some dry soils after 4 weeks provided suitable resuscitation methods are used in the preliminary stages of bacterial isolation. Moreover, *B. pseudomallei* will freeze-dry with a loss of up to 90% viable cells per freeze-dry cycle.

10.4 Ultraviolet Exposure.

Little has been done on the *B. pseudomallei* response to UV exposure (summarized in Table 2). One study claims only modest resistance to UV and greater susceptibility when compared with other bacterial species. *B. pseudomallei* were killed by UV rays at 465 $\mu W/cm^2$surviving for 7.75 min compared with other permanent soil bacteria that survived 1,860 $\mu W/cm^2$ for 31 min (Tong *et al.*, 1996). The experiment had limited objectives and very little numeric data, making it difficult to extrapolate its results to other settings. A starting inoculum of around 10^7 CFU/mL appears to have been used for each series. At present, there is little to suggest that *B. pseudomallei* has a significantly enhanced ability either to resist UV light or to repair the damage UV rays induce. The use of UV treatment units for some potable water supply systems should prompt an evaluation of the effect of UV on *B. pseudomallei* in liquid suspension.

10.5 pH Range.

More is known about the effects of environmental pH on *B. pseudomallei* (Table 2). A decade ago it was noted that the soils in which *B. pseudomallei* was most commonly isolated in the highly endemic region of northeast Thailand were unusually acidic

(Kanai *et al.*, 1994). Laboratory experiments showed that acid conditions enhanced the production of an acid phosphatase; now known to be a tyrosine kinase, which was thought to be a virulence factor (Kondo *et al.*, 1994a and 1994b). That line of inquiry quickly departed from adaptive bacterial responses to an acidic environment to follow the molecular basis of acid phosphatase expression (Burtnick *et al.*, 2001), leaving important questions unanswered. In our own series of acid exposure experiments, it was possible to show that *B. pseudomallei* tolerates an acidic pH quite well as far down as 4.5 at which point there appears to be a threshold phenomenon. Below this threshold, there is a divergence between the bacterial cells that are culturable by conventional means, and cells that are viable by supravital stain, suggesting the presence of viable but nonculturable bacteria (Figure 7). We have been able to recover some of these cells into a culturable form but do not know whether they are more, equally, or less virulent. The culturable cells are clearly potentially virulent. The significance of possible viable but nonculturable cells is that they may have either normal or even enhanced virulence, but will not be detected by conventional culture-based methods. Thus, those methods could underestimate the degree of risk in a contaminated environment. Experiments are needed to determine whether there might be a difference in virulence between acid stressed and unstressed *B. pseudomallei* in mice. Early data suggests that the acid stressed cells are at least as virulent. However, these results need to be confirmed in additional experiments. The VBNC (viable-but-not-culturable, see Introduction) bacteria take on a gram positive coccoid appearance (Table 1 and Figure 12), which reverts to the more conventional gram negative bacillary appearance on conversion to culturable bacteria. There appears to be less tolerance of alkaline conditions. Tong *et al.* (1996) confirmed a pH range of 5-8 for *B. pseudomallei*. The survival kinetics of *B. pseudomallei* as a function of acid exposure has not been obtained in detail. Our own data indicate that a pH of 4.5 or below causes a rapid fall in viable count (a 10,000-fold reduction or more, Figure 7). Recovery occurs only after removing the acidic medium and replacing it with fresh media at neutral pH .

10.6 Other Physical Stresses.

Several other physical stresses have been investigated (Tables 1 and 2). At temperature extremes (e.g., 0-4 °C and 42 °C), there are differences in bacterial survival patterns. Storing *B. pseudomallei* in the refrigerator (between 0 and 4 °C) converts a proportion of the bacteria to a nonculturable form. Loss of apparent viability can be 80-90% or more after 24 hr at 0 °C. At 42 °C, *B. pseudomallei* will grow rapidly in liquid media, exhaust the nutrients, and form a sediment of which around 80% are viable but not culturable. Once again, only a minority of bacterial cells remains culturable after 48 hr. While the effect of different temperatures on bacterial growth is quite well known, the effect of storage temperature on survival either in the laboratory or in the external environment is not well known. This information should be of considerable importance to assess the persistence of *B. pseudomallei* virulence. Even at 37 °C, growth of *B. pseudomallei* results in a significant discrepancy between the number of culturable and apparently viable cells. Therefore, it is clear that any predictive bacteriology analysis relying on numerical determinations will need to be performed under carefully controlled conditions.

Osmotic stress can also be used to demonstrate the possibility of VBNC bacteria. Over a range from 0.1 - 8 % w/v NaCl, the apparently VBNC bacterial cells were present at the higher and absent at the lower osmolarities (Figure 8). Again, these cells took on a gram positive coccoid form. No work has been done to study either the expression of potential virulence factors or the metabolic activity under these conditions. *B. pseudomallei*

survives exposure to solutions containing <2.5% NaCl w/v with no significant reduction in survival (i.e., with no discrepancy between colony count and flow cytometer viable cell count). Only at concentrations >2.5% w/v NaCl is there any evidence of the physiological stress also seen under low pH conditions. Less work has been done on seawater because, until recently, only a few cases of melioidosis had been associated with near drowning in sea water. However, the many reports of melioidosis following the recent Indian Ocean tsunami may provide epidemiological clues to a suitable experimental hypothesis.

10.7 Chemical Stress.

B. pseudomallei has been studied against only a small range of chemical exposures, mostly to assess the decontaminant effects of disinfectant solutions (Table 2). Chlorine is widely used to treat drinking water supplies at concentrations around 1 ppm (1 mg/L). Its activity against *B. pseudomallei* is of particular interest to water service providers in northern Australia. Preliminary studies showed that available chlorine was an effective decontaminant providing that the bacteria were in suspension and the source of available chlorine (e.g., hypochlorite solution) was correctly and recently formulated (Howard and Inglis, 2003). In the laboratory, concentrations of 10^3 ppm of available chlorine (i.e., 1 mL/L, formulated as hypochlorite solution) decontaminated 10^6 CFU of *B. pseudomallei* in stationary phase bacteria (NCTC 13177, and later, also confirmed with NCTC 10276). The same level of chlorine was used as a decontaminant without any breakthrough contamination in laboratory equipment (e.g., flow cytometer). A more detailed assessment of susceptibility to chlorine showed that different *B. pseudomallei* strains varied in their sensitivity to chlorine by over 100-fold. All strains showed an initial decrease to undetectable levels by conventional colony count. Tolerant strains were recoverable in culturable form after prolonged (30 min) chlorine contact time, followed by an enrichment broth step. Counts were conducted with large volume aliquots throughout the experiment to ensure that the count was <1 CFU/mL in all replicates. Some were able to tolerate up to several hundred parts per million. Tolerance was not a cumulative phenomenon. Prior exposure to 100 ppm available chlorine may produce a small additive effect similar to that observed with *Pseudomonas aeruginosa* (Sagripanti and Bonifacio, 2000) (Figure 9).

However, at the normal user concentration of 1 ppm available chlorine, it was possible to demonstrate viable *B. pseudomallei* after 30 min exposure to chlorine [by a flow cytometry method using a viability stain (SYTO-9 and propidium iodide labels from Molecular Probes) that distinguished live (apple green) from dead (brick red) bacteria], and then recover culturable bacteria after a period of further incubation. The ability of this species to tolerate the chlorination regimen used for routine drinking water disinfection is a matter for concern in some locations where chlorine treatment of the water supply can be sporadic and at variable concentration. The identification of the drinking water supply, as a likely source of *B. pseudomallei* in fatal melioidosis outbreaks, highlights the importance of chlorine treatment in melioidosis prevention (Inglis *et al.*, 1999; Currie *et al.*, 2001). The absence of further cases of melioidosis in one of those outbreak-affected locations, following introduction of rigorous chlorination of the water supply, is further evidence to support the melioidosis preventive role of water disinfection.

Further work is needed on improved chlorine delivery systems, and alternative disinfecting agents [e.g., DF200 foam, Decon Green (Sagripanti 2004, Sagripanti *et al.*, 2007)], chloramine (Howard and Inglis, 2005), cupric ascorbate, and several other products used for disinfection and decontamination (Sagripanti and Bonifacino, 1996, 1999, 2000).

Preliminary results indicate that chloramine may be one suitable alternative for disinfecting *B. pseudomallei*-contaminated water systems. But, tolerance, persistence, and recovery of these bacteria remain a possibility even in well managed systems due to sequestration in biofilms or within ameba. To our knowledge, DF200 foam and Decon Green have not been assessed on sequestered bacteria but only on bacteria in suspension or deposited on surfaces (Sagripanti *et al.*, 2007). Other disinfectants that need preliminary work include ozone and silver ionization-generating systems. Optimal procedures to decontaminate *B. pseudomallei* in aqueous suspensions or deposited on solid surfaces have not been defined.

11. METABOLIC ADAPTATION

Little is known about metabolic adaptation by *B. pseudomallei* to changes in its environment. The culture conditions used for initial recovery of clinical isolates and their subsequent identification by substrate utilization are not representative of the wide variety of conditions the species may encounter in its natural soil or water environment. While it is clear that this species has a wide range of potential carbon and nitrogen sources and strategies to ensure iron and phosphate supply, the metabolic range of *B. pseudomallei* is more restricted than *B. thailandensis*. It is clear from recent phylogenetic studies that *B. pseudomallei* is the more recent arrival and probably represents an adaptation to a more specialized habitat (Godoy *et al.*, 2003) (Figure 10). Perhaps that ecological development is reflected in the species' metabolic repertoire. There is no ability to fix atmospheric nitrogen; a feature required by *Burkholderia* species recently noted to reside in leguminous root nodules (Moulin *et al.*, 2001). The accumulation of PHB is a feature of a metabolism adapted to long-term survival; but, knowledge of its regulation is incomplete despite the recent publication of the *B. pseudomallei* genome (Holden *et al.*, 2004). Prominent PHB granules are formed in each bacillus (Figure 11). The PHB accumulation is affected by carbon: nitrogen (C:N) balance. Changes in this C:N ratio will affect the relative amount of PHB synthesized. Preliminary studies on the expression of cellular fatty acids show only one subtle difference between *B. pseudomallei* and *B. thailandensis*; the presence of small quantities of 2-hydroxymyristic acid (2-HMA) in *B. pseudomallei* (Inglis *et al.*, 2003). There is no evidence of significant variation in 2-HMA production with increasing age of bacterial colonies. Regulation of metabolic activity in response to changing environmental conditions has not been investigated. Early studies with gene arrays open up some interesting new approaches to assessing the activity of the *B. pseudomallei* genome. The production of 2-HMA is one metabolic pathway worth pursuing in depth because a similar product has been identified in *Salmonella typhimurium,* which is thought to assist intracellular survival and thus pathogenesis (Gibbons *et al.*, 2000).

12. VIABLE BUT NON-CULTURABLE BACTERIA (VBNC)

We have noted a divergence between culturable *B. pseudomallei* and apparently viable bacterial cells that cannot be cultured by conventional means in several experimental systems. Low pH and high osmolarity produce this result. The practical importance of this effect is that culture-based detection will, by definition, fail to recognize the presence of a pathogen that is nonculturable without additional laboratory manipulation If the pathogen is still viable and potentially pathogenic, detection systems that do not rely on culture-based methods will have to be used. A viable but nonculturable state was proposed to explain the behaviour of sublethally damaged marine bacteria around a decade ago

(Weichart and Kjelleberg, 1996), and has attracted controversy on the grounds that it appears self-contradictory (Barer and Harwood, 1999). The argument over semantics will not be reiterated here. Before a VBNC state can be claimed for *B. pseudomallei* with any degree of certainty, more work is needed on cellular behaviour (e.g., cell elongation, continuing metabolic activity, ultrastructural integrity) and gene expression during conditions of either environmental stress or starvation. *Legionella pneumoniae* was recently shown to have an adaptive pathway for these conditions (Faulkner and Garduno, 2002). Given the parallels with many aspects of Legionella biology, *B. pseudomallei* may eventually be shown to behave in a similar way. The morphological variations noted in response to either physical or chemical stress are interesting At ultrastructural level, the outer surface of the coccoid forms appears corrugated, consistent with a condensation of cellular mass (Figure 12). Cross sections of these cells at transmission electron microscopy (TEM) do not show major qualitative changes in either the internal structure or the cell envelope, though there was variation in cell diameter. *B. pseudomallei* in stationary phase culture comprises short bacilli measuring around 1.2 µm. Detailed measurements do not appear to have been published. After acid stress, coccoid forms may reach 2-3 µm and are occasionally much larger. There is a spectrum of sizes measured by flow cytometry in a given bacterial population (Figure 13). Flow cytometer assessment of acid-stressed *B. pseudomallei* stained with the supravital dye combination of SYTO and propidium iodide indicates that a major fraction of these bacteria are intermediate between clearly viable and clearly nonviable. Therefore, the dye-based approach has limitations that cannot be resolved by this method (Boulos *et al.*, 1999).

The VBNC issue also has implications for our understanding of virulence. If VBNCs are truly living, they should be capable of causing disease. If they are dead, the only damage they will be capable of producing should result from bacterial toxic products incapable of further replication. Bacterial cells in the poorly defined zone between fully viable and fully nonviable could, in theory, exhibit a variety of behaviour patterns, including either enhanced or depressed virulence. The VBNCs (generated in suspension by exposure of *B. pseudomallei* to 10 ppm chlorine for 30 min) were inoculated in a BALBc mouse model of infection (Ulett *et al.,* 2000). Results indicate that the VBNC cells are still capable of causing infection at a similar dose to culturable *B. pseudomallei,* although these data require confirmation. Further studies are needed to determine whether expression of critical virulence factors occurs due to prior environmental conditioning or physicochemical stress. The mouse system provides an experimental model to assess the impact of changing environmental conditions on encounters between *B. pseudomallei* and mammals.

13. SURVIVAL HABITATS OR NICHES PROVIDING ENHANCED SURVIVAL

B. pseudomallei may not persist in a monodispersed suspension in the wild. However, data on its preferred microhabitat is lacking. Normally, *B. pseudomallei* is found in surface water, soil, or a mixture of the two. In soil, *B. pseudomallei* is normally located in the rhizosphere or root zone where a complex biota exists. Biofilm of one or more species of soil bacteria can develop at any naturally occurring interface between liquids and solids. This interface is a preferred habitat for biofilm-formation by B. ps*eudomallei* (Levy *et al.*, 2003).

B. pseudomallei forms a film of bacteria at the liquid/air interface in a container of broth after incubation at 37 °C for 24 hr or more. A similar film of stationary phase bacteria forms at liquid/solid interfaces and is known as a biofilm. Biofilms contain slowly growing bacteria and extracellular polysaccharide in complex 3-dimensional structures; and their growth or lack thereof is controlled by release of quorum-sensing substances (acyl homoserine lactones) (Song *et al.*, 2005). Slow-growing bacterial consortia are inherently more resistant to the action of disinfectants and antibiotics, and this form of growth has been cited as a possible explanation for *in vivo* resistance to antibiotics used to treat *B. pseudomallei* infections (Vorachit *et al.*, 1993).

13.1 Protozoa.

Penetration of free-living amoebae by *B. pseudomallei* uses the coiling phagocytosis mechanism seen with other intracellular bacteria [e.g.,Legionella and Listeria (Inglis *et al.*, 2000)]. Entry into Acanthamoeba trophozoites forms vacuoles full of bacteria, and when conditions are right may lead to formation of amoebic cysts containing live bacteria. Persistence in amoebic cysts represents another means of surviving very hostile environmental conditions. Amoebic species in which *B. pseudomallei* can survive include *A. astronyxis, A. castellani, A. palastinensis,* and *A. polyphaga.* By analogy with Legionella, *B. pseudomallei* should also survive in *Hartmanella vermiformis* and a variety of other free-living species (Wadowsky *et al.*, 1991). Many of these protozoa graze on the polymicrobial biofilms that form in untreated wet habitats. A cycling and recycling of facultative intracellular bacteria, including *B. pseudomallei,* by free-living amoebae is envisaged. The effect on cellular virulence by amoebic passage of these bacteria has yet to be studied. A similar process in Legionella is known to enhance cellular virulence (Cirillo *et al.*, 1999). In preliminarily studies in Western Australia, *B. pseudomallei* DNA has been detected by a PCR-based method inside amoebae isolated from surface water bodies in the tropical north of the state, near to locations where human melioidosis has occurred. Although this highlights the possible link between an environmental source and human disease, it is only a circumstantial link. The presence of DNA does not equate with the persistence of viable, culturable bacteria. It is possible that the amoebae in question had fully digested small quantities of *B. pseudomallei* and rendered the bacteria completely innocuous. Nevertheless, this issue needs elucidation since small bacillus-filled amebic vacuoles are about 5-15 µm in diameter, which is close to the ideal size of aerosolized particles that can efficiently infect humans via the respiratory tract. As in the case of Legionnaire's disease, the infective particle and the normal intracellular habitat may be the same entity (Brieland *et al.*, 1997).

13.2 Fungi.

Another environmental cellular habitat recently identified for *B. pseudomallei* is the cytoplasm of arbuscular mycorrhizal fungi belonging to the genus *Gigaspora* (Levy *et al.*, 2003). Penetration, persistence and survival of *B. pseudomallei* in culturable form within fungi were all demonstrated in this study. Mycorrhizal fungi form endosymbioses with plant roots, and therefore provide an intracellular habitat for bacteria, inside another eukaryotic habitat. This double layer of protection shields bacteria from the external environment and provides a potential genetic crossroads for traffic between different kingdoms. The periodic acquisition of genetic material from either the host fungus or plant from contained *Burkholderia* could explain the genetic complexity of the *Burkholderias*.

13.3 Legumes.

Legumes are nitrogen fixing plants whose root nodules contain symbiotic bacteria that are usually associative (atmospheric) nitrogen fixers. Recently, members of the genus *Burkholderia* have been noted to be capable of fixing atmospheric nitrogen in root nodule formations (Moulin *et al.*, 2001). However, *B. pseudomallei* is not one of the atmospheric nitrogen-fixing *Burkholderia* members. Despite a concerted search, we did not find *B. pseudomallei either* on or in digests of roots from native legumes in northern Australia. However, *B. pseudomallei* has been recovered from the soil adhering to the roots of a native wattle; *Acacia colei.* (Inglis *et al.,* 2000b). This finding is consistent with root surface colonization but does not support an established commensal or symbiotic relationship. On the other hand, *Burkholderia vietnamiensis* has been isolated after surface sterilization of *A. colei* seeds from Western Australia. The latter species does fix atmospheric nitrogen.

14. CONCLUSIONS

The implications of the above considerations should be clear to those either working with *B. pseudomallei* or those responsible for protecting the public from melioidosis. The key to protect civilian and military personnel from melioidosis is to avoiding exposure. The use of personal protective equipment can be effective when the location and timing of exposure is predictable (e.g., servicemen on exercise in a known disease hot zone).

The metabolic behavior of *B. pseudomallei* in its natural habitat (either inside eukaryotic cells or in polymicrobial biofilms) is likely to differ from its behaviour in conventional, rapid-growth laboratory culture conditions. Metabolic adaptation and internalization within eukaryotic systems (e.g., unicellular, plants, etc.) likely provide extraordinary environmental advantages to *B. pseudomallei.* Therefore, understanding the environmental biology of *B. pseudomallei* should lead to improvements in control and prevention of melioidosis. Study of the *B. pseudomallei* metabolome and its regulation should help explain aspects of environmental survival and cellular pathogenesis.

Detection of environmental *B. pseudomallei* can be very challenging when the viable fraction is low. In this respect, *B. pseudomallei* represents one of the more difficult organisms to detect and study among the nonspore-forming bacteria. The development of polyphasic bacterial detection systems that accurately identify the presence of live bacteria should allow much more rigorous evaluation of survival and decontamination of *B. pseudomallei*.

The contribution of eukaryotic life forms in *B. pseudomallei* environmental persistence and survival explains why chemical decontaminants (e.g., hypochlorite) can be less effective against this organism than against Enterobacteriaceae in the field. The data discussed above indicates that more effective decontamination strategies need to be developed against *B. pseudomallei*, particularly to protect drinking water systems.

The unusually long environmental persistence in soil, water, as well as the resistance and tolerance to physical factors, pH changes, osmolarity, and chemicals (e.g., hypochlorite) makes treatment and remediation of environments contaminated with

B. pseudomallei extremely challenging. Furthermore, remediation of contaminated soils needs further evaluation of the persistence of *B. pseudomallei* either associated to leguminous plants or in the rhizosphere.

While it is possible that free-living amoebae and other protozoa could assist as a bacteria-scavenging remediation system, protozoa could potentially enhance the risk of *B. pseudomallei* by passage-enhanced bacterial virulence.

B. pseudomallei will likely remain a pathogen of interest in bio-defense since detection is difficult; treatment of melioidosis is long; relapse is frequent; and a vaccine is lacking.

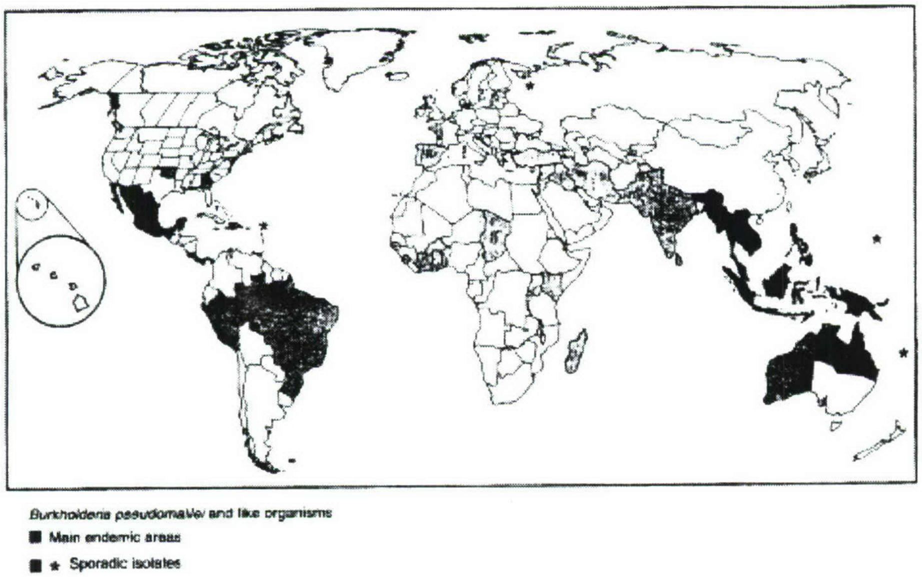

Figure 1. Melioidosis World Distribution. The main endemic areas are darkly shaded. Disease distribution in the Americas is controversial, and its existence in Africa is doubted.

Figure 2. Map of Southeast Asia Showing Locations where Melioidosis is Known to Occur. The distribution of the disease is unknown in Indonesia. Information on most locations throughout the region is patchy. Except, possibly for Singapore, no nationwide distribution data are available.

Figure 3. Map of Brazil Showing Known (red) and Likely (yellow) Locations of Melioidosis since 2003.

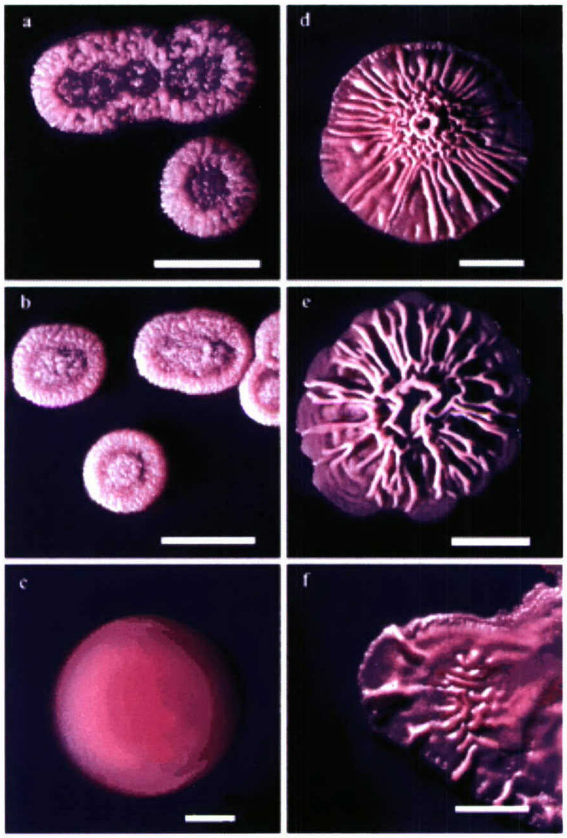

Figure 4. Mucoid Colony Variant of *B. pseudomallei* (Shown in Figure 4e). (Howard and Inglis, 2003).

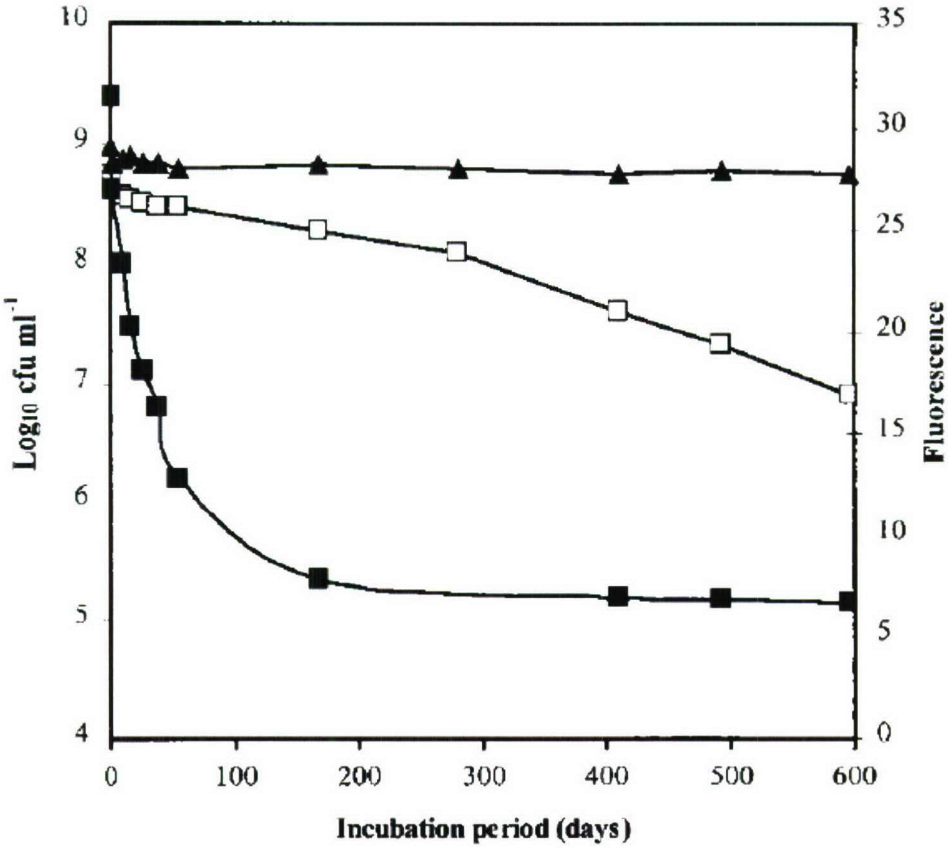

Figure 5. *Legionella pneumophila* in Tap Water, Showing Relationship between Total Cell Count (closed triangles), Plate Count (open squares), and Nile Red Fuorescence (closed squares), which Measures PHB (Source: James et al., 1999).

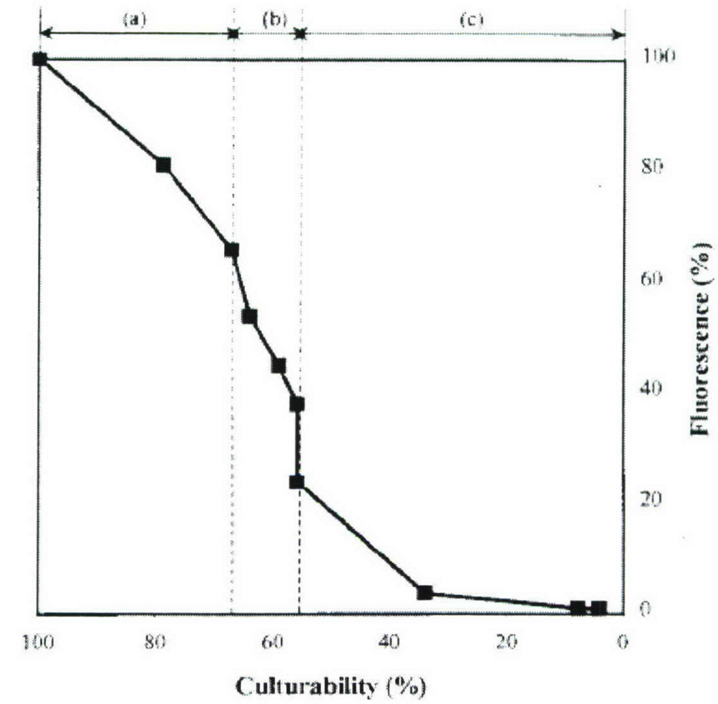

Figure 6. Relationship between PHB (Fluorescence) and Culturability of *Legionella pneumophila* in the Same Study. (Source: James et al., 1999).

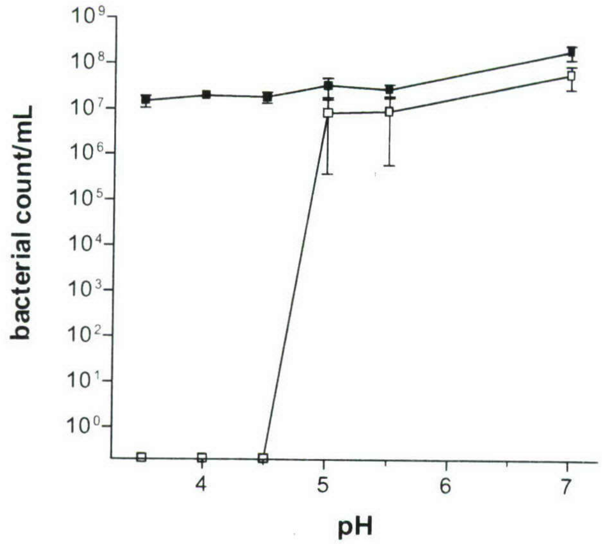

Figure 7. Graph Showing Divergence between *B. pseudomallei* Colony Count and Viable Cells as Detected by Flow Cytometry and Supravital Stain at Low pH. Open boxes indicate the bacterial count in colony-forming units per milliliter. Closed boxes indicate the viable bacterial count detected by flow cytometry.

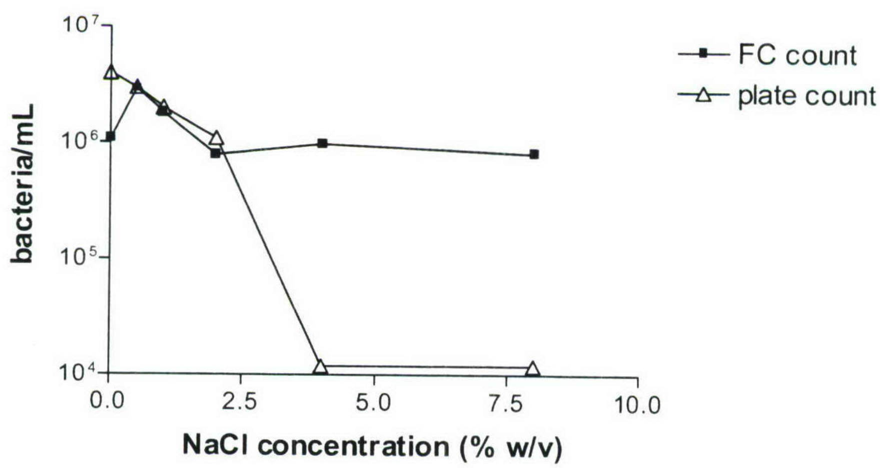

Figure 8. *B. pseudomallei* NCTC 13177 at Varying NaCl Concentrations (w/v) Showing Divergence between Plate Count (CFU/mL) and Flow Cytometer Viable Count.

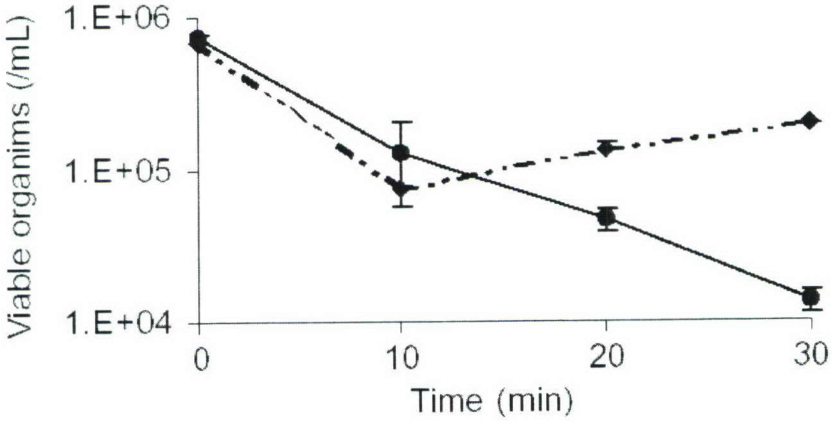

Figure 9. Survival of *B. pseudomallei* NCTC 13177 with no Prior (circles) and Prior (diamonds) Exposure to 100 ppm Chlorine after Subsequent Exposure to 1 ppm Chlorine. Error bars = standard error of the mean (Source: Howard and Inglis, 2003)

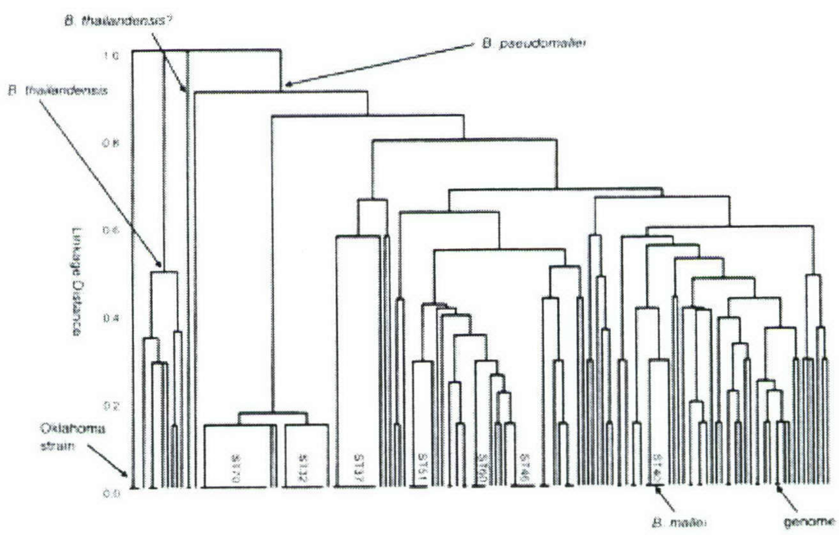

Figure 10. Phytogenetic Tree of the *Burkholderias (Source:* Godoy *et al*, J. Clin. Microbiol. 2003).

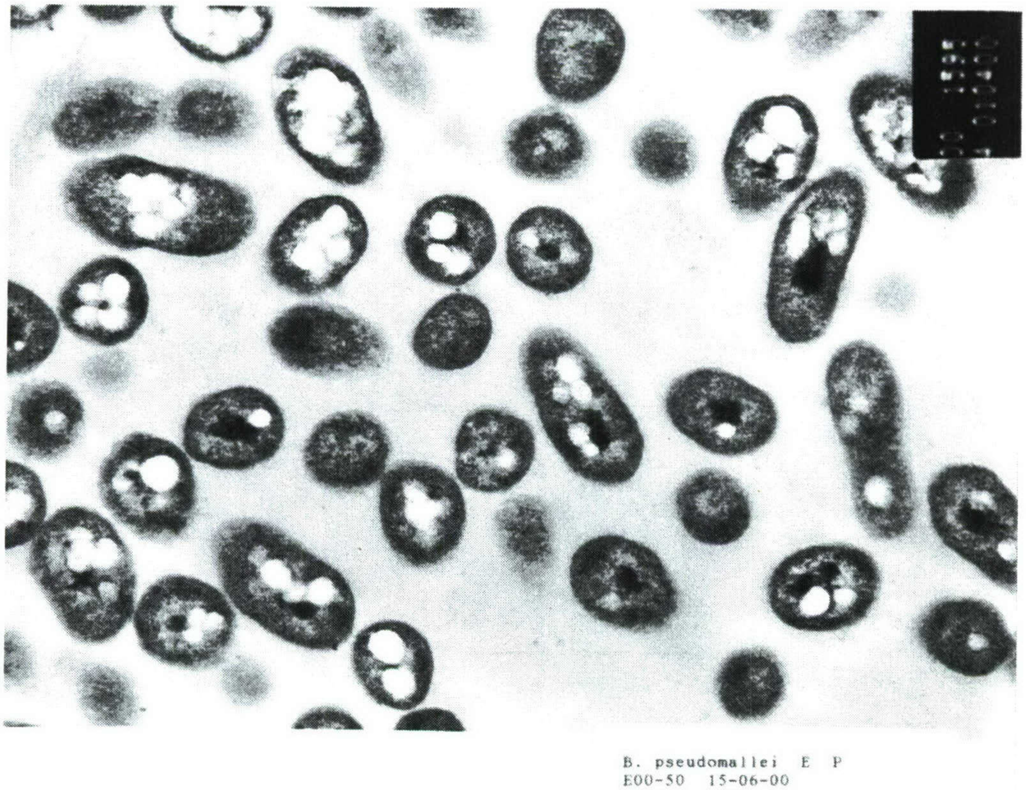

Figure 11. Transmission Electron Microphotograph of *B. pseudomallei* Showing Prominent Intracellular Inclusions of Polyhydroxybutyrate (Source: White, 1989).

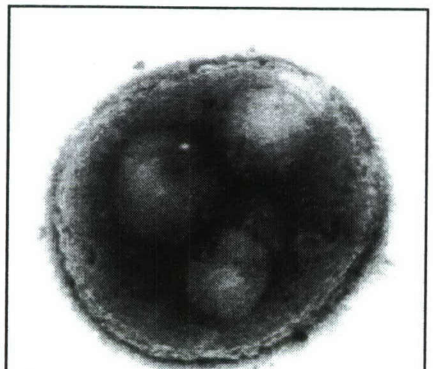

Figure 12. Transmission Electron Photomicrograph of Coccoid *B. pseudomallei* Cell after Physical Stress. The diameter of the cell is approximately 0.5 μm.

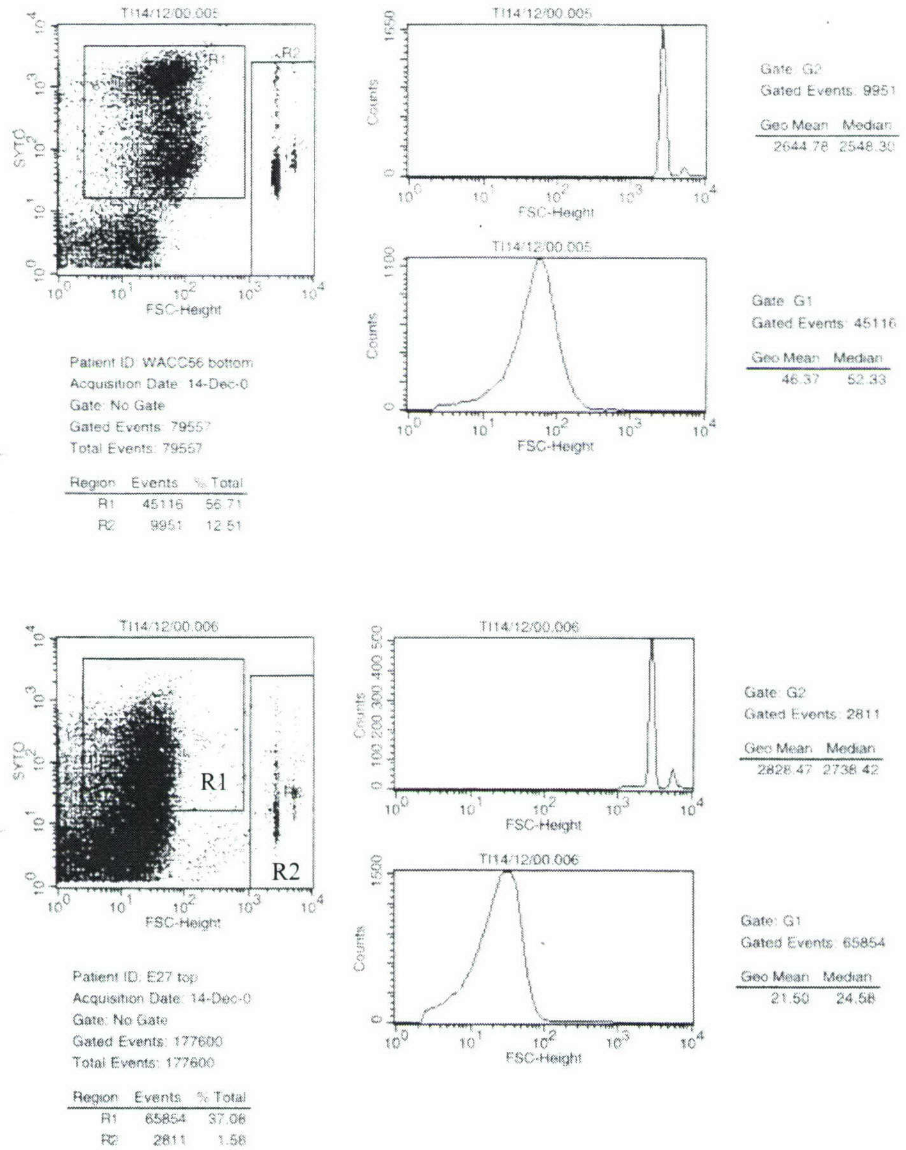

Figure 13. Derivation of Bacterial Count by Flow Cytometry. *B. pseudomallei* NCTC 13177 (WACC 56) Above; with *B. thailandensis* E 27, Below. Particles with size and optical properties of SYTO-stained bacteria in box R1 have been compared with polystyrene bead size and count standard in box R2. Size distribution histograms of the two boxes are shown to the right of each output record, labeled G1 and G2, respectively. Particles (10,000) were counted and compared to a 1-µm diameter polystyrene reference particle suspension of 10^6/mL.

Table 1. Effect of Varying Conditions of Growth on *B. pseudomallei* NCTC 13177 Following Inoculation of 10^6 Washed Bacteria

Conditions	Plate count/mL	Cytometer count/mL	Comments
TSB @ 37 °C	3.2×10^6	3.6×10^7	Bright green bacilli
TSB @ 0 °C	4.4×10^5	4.9×10^6	Green, some red bacilli
TSB @ 42 °C	1.6×10^6	2.7×10^7	Green, red mixture
TSB @ 37 °C, pH 4.3	7.0×10^3	1.1×10^6	Mainly yellow bacilli
TSB @ 37 °C, AnO2	4.2×10^6	1.5×10^8	Mainly green bacilli
SDW @ 37 °C	9.4×10^5	3.8×10^7	Red, yellow mix
TSB @ 37 °C, 1 wk pellicle	1.0×10^7	2.7×10^8	Short green bacilli, few red
TSB @ 37 °C, 1 wk sediment	3.1×10^6	1.7×10^8	Mainly red, yellow bacilli
SDW @ 2 °C x 12m	$<7 \times 10^3$	6.8×10^8	Green cocci, variable size
TSB @ 37°C, AnO2 x 9m	1.6×10^5	7.4×10^6	Red, yellow variable shape

Table 2. Effect of Environmental Factors on the Survival of *B. pseudomallei*.

Environmental Factors	Conditions	Duration of Experiment in Days, Minutes, or Hours	Outcome
Distilled Water[1]	Triple distilled 10^6 CFU/mL	>3 years	Rise to 10^8 CFU/mL after 1 month, then fall to 10^4 CFU/mL after 2 years
Water Content[2]	0%	30 days	No later survival
	5%	40 days	No later survival
	10%	70 days	No later survival
	20%	439 days	No later survival
	40%	>726 days	Survived for the experiment duration
	80%	>726 days	Survived for the experiment duration
Temperature[2]	0°C	<42 days	No later survival
	8°C	<190 days	No later survival
	16°C	<477 days	No later survival
	24 °C	>720 days	Survived for the experiment duration
	32°C	>720 days	Survived for the experiment duration
	40°C	<42 days	No later survival
	48°C	<12 days	No later survival
pH	(2) 2	1 day	No later survival
	(2) 3	7 days	No later survival
	(2) 4	231 days	No later survival
	(2) 5 – 8	>726 days	Survived for the experiment duration
	(2) 9	26 days	No later survival
	(2) 10	1 day	No later survival
	(3) 3.5 – 7.0	24 hr	7 $\log_{10}$ reduction at pH <4.5
Chlorine	(3) 1 mg/L	30 min	4 $\log_{10}$ reduction in CFU/mL
	(4) 0.5 – 4.0 mg/L (4) 4 mg/L (pH 6-9)	0-60 min Up to 30 min	1-6 $\log_{10}$ reduction (at pH 9 to pH 6, respectively) 7 $\log_{10}$ reduction after 30 min
Chloramine	(3) 1 mg/L	24 hr	2.5 $\log_{10}$ reduction in CFU/mL
		48 hr	3 $\log_{10}$ reduction in CFU/mL
UV Light (254 nm)	(2) 4.65 W/m^2	7.75 min	Shorter survival than control bacteria
	(3) 44 J/m^2 (3) 120 J/m^2	N/A	1 $\log_{10}$ reduction in CFU/mL 4 $\log_{10}$ reduction

Sources: [1] Wuthiekanun et. al., 1995 (87); [2] Tong et. al., 1996 (78); [3] Howard and Inglis, 2005 (25); [4] Thomas and Forbes-Faulkner, 1981 (77).

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